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Research

The Effects of 2,4-Dichlorophenoxyacetic Acid on The Induction of Callus from Cotyledon and Hypocotyl Explants of Butterfly Pea (*Clitoria ternatea*)

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ABSTRACT

Clitoria ternatea (Butterfly pea) is a tropical medicinal and fodder legume from the Fabaceae family possessing various beneficial phytochemical compounds linked to the mammalian neuroprotective mechanism. Callus and cell suspension cultures are excellent alternatives for harnessing secondary metabolites from medicinal plants. The current study aims to induce callus from cotyledon and hypocotyl explants of *C. ternatea* for the establishment of cell suspension cultures. Cotyledon and hypocotyl explants from two-weeks-old seedlings were subjected to half-strength MS medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) at different concentrations (0.5 mg/L to 2.5 mg/L) and callus scoring and morphology were assessed at week 8 of culture. Results revealed that the treatment of 0.5 mg/L 2,4-D resulted in the highest percentage of callus induction (100%) and the highest callus scoring for both cotyledon and hypocotyl explants with friable callus morphology. Cotyledon explants exhibited a higher callus scoring with a relative value of 3.03 ± 0.20 compared to hypocotyl explants at 1.80 ± 0.12. This study thereby provides a basis for future studies on callus induction studies and the establishment of cell suspension cultures of *C. ternatea* for the production of valuable secondary metabolites linked to the memory enhancing properties of the plant.

Key words: 2,4-Dichlorophenoxyacetic acid, callus induction, *Clitoria ternatea*, cotyledon, hypocotyl

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INTRODUCTION

Clitoria ternatea commonly known as the butterfly pea plant, is a perennial herbaceous, dicotyledon plant from the Fabaceae family and is native to Asia (Al-Snafi, 2016). It bears vivid deep-blue or white pentamerous zygomorphic pea-shaped flowers with a tubular calyx consisting of five free petals which bloom all year long (Taur *et al*., 2010). The utilization of *C. ternatea* as folk medicine is gaining popularity as every part of this plant including the flowers, leaves, roots, and seeds are nutritious and serve as traditional medicine in the form of health tonics and supplements (Mukherjee *et al*., 2008; Al-snafi, 2016).

Clitoria ternatea is esteemed as an essential tropical medicine owing to its abundance of phytochemical compounds (Mukherjee *et al*., 2008). This plant contains phytochemical compounds such as pentacyclic triterpenoids (taraxerol, taraxerone), alkaloids, anthocyanins, flavonoids, tannins, saponins, resins, glycosides, phenols, and steroids (Chauhan *et al*., 2012; Manjula *et al*., 2013; Al-snafi, 2016; Lijon *et al*., 2017; Lee *et al*., 2021) associated with pharmacological properties of nootropic, anxiolytic, analgesic, antipyretic, sedatives, anticonvulsant, anti-inflammatory, antidepressant, antioxidants, and others (Taranalli & Cheeramkuzhy, 2000; Devi *et al*., 2003; Gomez & Kalamani, 2003; Jain *et al*., 2003; Mukherjee *et al*., 2008). Particularly, pentacyclic triterpenoids such as taraxerol and taraxerone are the dominant phytochemical compounds that exist in *C. ternatea* (Lijon *et al*., 2017) which demonstrated a wide spectrum of pharmacological properties, including being anti-cancer, antimicrobial, anti-inflammatory, and anti-acetylcholinesterase activities (Takasaki *et al*., 1999; Lin *et al*., 2001; Singh *et al*., 2002; Jang *et al*., 2004; Lee *et al*., 2004; Naik *et al*., 2004). Taraxerol, a valuable pentacyclic triterpenoid, found mainly in the roots of *C. ternatea* has been proven to enhance and increase acetylcholine content in the brain, thus functioning as a memory protectant to memory-impaired rats (Kumar *et al*.,

2008). This further confirms the potential of *C. ternatea* root extracts as a memory enhancer in dementia or disorders related to loss of learning ability and Alzheimer's (Rai, 2010). These bioactive compounds were linked to memory-enhancing properties and therefore, could serve as a promising alternative to improving learning and memory performance (Kumar *et al*., 2007; Lee *et al*., 2021). Apart from serving as a medicinal plant, this plant too functions as an ornamental plant due to its dazzling flower colour (Gomez & Kalamani, 2003). The blue flowers of this plant have been widely used as a natural food colourant in traditional Southeast Asian dishes, desserts and beverages.

The emerging commercial value of the phytochemical compounds from this plant species has prompted a surge in demand which causing increasing pressure on the plant density (Al-Asmari *et al*., 2020). Plant tissue culture techniques offer an efficient alternative in the propagation of valuable plant species for in-depth study on plant response and the accumulation of natural secondary metabolites. As such, *in vitro* callus culture is an ideal approach to isolate the phytochemical compounds while minimizing the issues of over-harvesting of *C. ternatea* (Rai *et al*., 2022). Callus exhibit totipotency consisting of the full genetic information of the plant. The totipotent nature of plant cells enables rapid multiplication of true-to-type clones that possess similar genetic information from the mother cells (Verdeil *et al*., 2007). Therefore, callus produced from the cotyledon and hypocotyl explants can be induced to produce novel secondary metabolites as expressed in certain organs of the plant. To date, callus and cell suspension cultures are broadly employed in producing valuable phytochemical compounds from medicinal plants such as *Artemisia annua* L., *Centella asiatica* L. urban (Pegaga), *Eurycoma longifolia* (Tongkat Ali), *Panax quinquefolium* (American ginseng), where these techniques have been proven to be efficient in producing the phytochemical compounds as compared to other *in vitro* techniques (Chan *et al*., 2010; Tan *et al*., 2010; Nhan & Loc, 2017; Kochan *et al*., 2019). Of these, callus cultures are particularly reliable in stimulating and producing therapeutically beneficial phytochemical compounds from plants, whereas cell suspension cultures offer mass production of valuable compounds (Ogita, 2015; Efferth, 2019). Previous *in vitro* studies on *C. ternatea* involves the regeneration of the plant, callus induction, root induction, and adventitious root cultures using different types of explants such as the leaf, cotyledon, hypocotyl, and nodal (Mohamed & Taha, 2011; Chan *et al*., 2017; Mishra *et al*., 2019; Lee *et al*., 2021). Up to now, a few studies have documented *in vitro* cultures of *C. ternatea,* and not much is being reported on callus cells being harvested for the production of novel secondary metabolites of this plant. The current study aims to induce callus from cotyledon and hypocotyl explants from *in vitro* seedlings of *C. ternatea* as an attempt to establish cell suspension cultures for future studies on the production of novel pentacyclic triterpenes.

MATERIALS AND METHODS

Plant Material

Seeds from the mature seedpods of the *Clitoria ternatea* plant were obtained from the mother plants grown at the Herbarium Unit of the School of Biological Sciences, Universiti Sains Malaysia. The seeds acquired were surface-sterilized based on Lee *et al*. (2021) with modifications in which the seeds of *C. ternatea* were rinsed thoroughly under running tap water for 15 minutes prior to mild agitation in 60% v/v Clorox© (bleach) solution containing two drops of Tween 20 for 10 minutes and sterilized by gentle agitation in 70% v/v ethanol for 1 minute. The seeds were then rinsed using autoclaved distilled water thrice, followed by drying on sterilized filter paper. The surface-sterilized seeds were inoculated onto hormone-free half-strength Murashige and Skoog (½ MS) (Murashige & Skoog, 1962) media with 1.5% (w/v) sucrose, 0.8% (w/v) plant agar powder (Duchefa Biochemie B.V., The Netherlands) and incubated under white fluorescent light (Philips TLD, 36 W. 150 µmol $m^{-2}s^{-1}$) with 16 hours photoperiod at the temperature of 25 ± 2 °C for the establishment of sterilized seedlings. The cotyledons and hypocotyls of the two-week-old *in vitro* seedlings were excised into the sizes of 0.6 × 0.6 cm^2 and 1 cm, respectively, prior to hormone treatments in the subsequent experiments.

Callus induction

The *in vitro* cotyledon and hypocotyl explants were inoculated in half-strength MS media supplemented with 2,4-D at the concentrations of 0.5 mg/L, 1.0 mg/L, 1.5 mg/L, 2.0 mg/L, and 2.5 mg/L. The half-strength MS media contained 1.5% (w/v) sucrose and was solidified with 0.8% (w/v) plant agar powder (Duchefa Biochemie B.V., The Netherlands). The experiments were repeated thrice, with each treatment consisting of 18 replicates with three explants per replicate. The cultures were maintained under white fluorescent light (Philips TLD, 36 W. 150 µmol $m^{-2}s^{-1}$) with 16 hours photoperiod at a temperature of 25 ± 2 °C for 8 weeks.

Data collection and statistical analysis

Parameters such as callus morphology (texture, colour), percentage of callus induction, and qualitative callus scoring (Figures 1 and 2 as a guide) were recorded on the 8th week upon incubation. The data collected were subjected to statistical analysis via IBM Statistical Package for the Social Sciences (SPSS) software version 27 using one-way analysis of variance (ANOVA) at 95% significance level and Tukey HSD (Honest Significant Difference) test at the significance level of $p \leq 0.05$.

RESULTS AND DISCUSSION

The current study thereby revealed that 0.5 mg/L 2,4-D was the optimal treatment for inducing friable callus from the cotyledon and hypocotyl explants as callus induction rate of 100% was achievable. Next, 0.5 mg/L 2,4-D treatment managed to yield the highest callus scoring of 3.03 ± 0.20 in cotyledon explants and 1.80 ± 0.12 in hypocotyl explants, respectively. However, in terms of callus scoring for the 2,4-D treated cotyledon

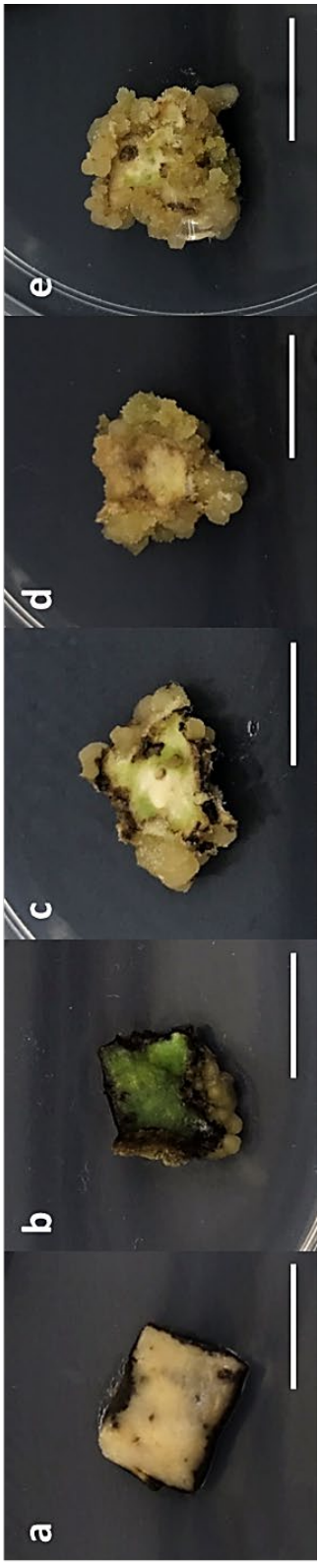

Fig. 1. Callus scoring at different levels for cotyledon explants. **(a)** 0, **(b)** 1, **(c)** 2, **(d)** 3, **(e)** 4. The scale bars represent 1 cm.

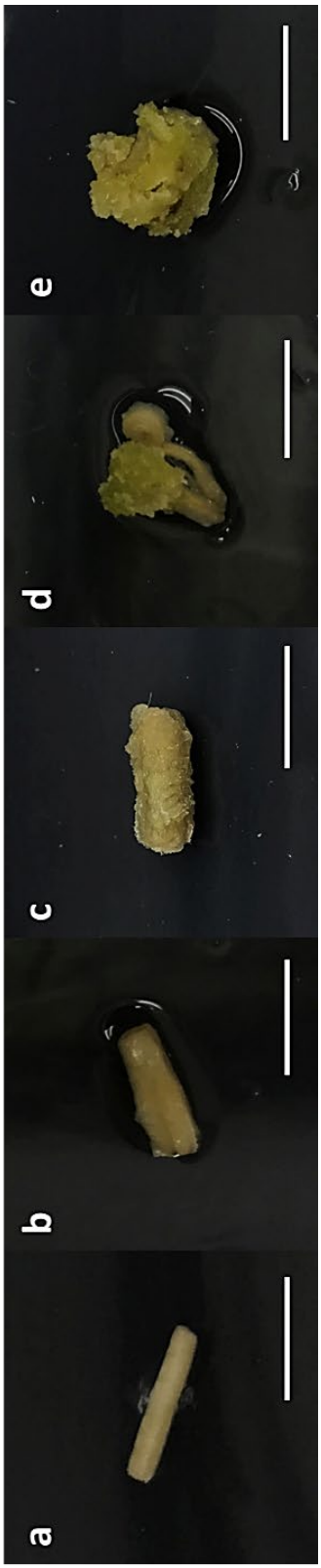

Fig. 2. Callus scoring at different levels for hypocotyl explants. **(a)** 0, **(b)** 1, **(c)** 2, **(d)** 3, **(e)** 4. The scale bars represent 1 cm.

explants, there were no significant differences observed between the treatment of 0.5 mg/L and 2.5 mg/L 2,4-D (Table 1). As for the 2,4-D treated hypocotyl explants, the results obtained in terms of callus scoring showed no significant difference between 2,4-D treatments, but there were significant differences observed between the control and the treatments in 2,4-D (Table 2). The type of callus obtained from both types of explants was friable, soft, watery, sticky, and yellowish-brown in colour. To date, efficient callus induction protocols for medicinal plants such as *Astragalus missouriensis* Nutt., *Eysenhardtia polystachya* and *Sophora alopecuroides* Linn. have been successfully developed, and the potential applications of *in vitro* callus culture have led to increased production of phytochemical compounds (Ionkoya, 2009; Bernabé-Antonio *et al*., 2017; Senthil, 2020). *In vitro* callus culture on *C. ternatea*, on the other hand, has yet to be investigated. However, Lee *et al*. (2021) discovered that 2,4-D could induce callus from *C. ternatea* cotyledon explants whereas Chan *et al*. (2017) observed callus formation from cotyledon and hypocotyl explants of *C. ternatea* in the treatments of indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA). However, these studies did not indicate the callus morphology and type of callus obtained. Consequently, detailed callus induction and proliferation studies are required for the establishment of callus and cell suspension cultures of medicinal plants such as *C. ternatea* especially for the evaluation of its secondary metabolites. In the current study, half-strength MS media was applied for all of the treatments, similar to media strength from Lee *et al.* (2021) and Chan *et al.* (2017). Half-strength MS media have also been proven to be the ideal media for the seedling development of Bambara groundnut (Fabaceae) (Koné *et al*., 2015) and for the callus induction of *Ulex europaeus* (Fabaceae) (Ramírez *et al*., 2012). Moreover, Ramírez *et al*. (2012) found that phenotypes obtained *in vitro* were more similar to those obtained from field-grown plants via half-strength MS media for *Ulex europaeus* (Fabaceae).

Table 1. The regeneration effects of different concentrations of 2,4-D on cotyledon explants of *C. ternatea* after 8 weeks of culture in half-strength MS media

2,4-D concentrations (mg/L)	Percentage of callus induction (%)	Callus scoring (Relative value)	Callus texture	Callus colour
0.0 (Control)	0.00 ± 0.00^{b}	0.00^{c}	N/A	N/A
0.5	100.00 ± 0.00^{a}	3.03 ± 0.20^{a}	Friable, soft, watery, and sticky	Yellowish-brown
1.0	88.24 ± 8.05^{a}	1.88 ± 0.24^{b}	Friable, soft, watery, and sticky	Yellowish-brown
1.5	85.19 ± 7.73^{a}	1.63 ± 0.21^{b}	Friable, soft, watery, and sticky	Yellowish-brown
2.0	90.20 ± 6.86^{a}	2.11 ± 0.24^{b}	Friable, soft, watery, and sticky	Yellowish-brown
2.5	88.89 ± 6.60^{a}	2.22 ± 0.25^{ab}	Semi-friable, hard	Yellowish-brown, greenish

The data present mean ± SE. Mean values followed by the same alphabet were not significantly different (Tukey HSD test at $p \leq 0.05$).

In this study, after one week of inoculation, the 2,4-D treated cotyledon and hypocotyl explants started to swell and expand. Callus development was detected in the fourth week from the excision sites of both the cotyledon and hypocotyl explants. 2,4-D is the commonly used synthetic auxin in inducing callus as it could revert cells in the explant to enter dedifferentiation state (George *et al*., 2008). With reference to Ikeuchi *et al*. (2013), the addition of exogenous auxin such as 2,4-D would stimulate the auxin response factor (ARF) transcription factors to activate the expression of lateral organ boundaries domain (LBD) family transcription factors, thereby induce E2 promoter binding factor 'a' (E2Fa) which takes part in cell cycle reentry, and lead to callus formation. Hence, under the treatments of 2,4-D, callus formation commenced as tissues within explants began to de-differentiate in order to produce meristematic and actively proliferated cells, thereby resulting in explant tissue swelling (George *et al*., 2008; Mastuti *et al*., 2017). As the cycle continues, actively divided cells then lead to callus development (George *et al*., 2008; Mastuti *et al*., 2017). In addition, auxin has been shown to increase cell extension by inducing the acidification of the cell wall, permitting the cell to absorb water and resulting in swelling (Stals & Inzé, 2001; Richard *et al*., 2002; Sado *et al*., 2014), which is in consistent with the findings of this study whereby swelling of explants was evident. Next, studies had shown that addition of 2,4-D could effectively aid in callus induction of other Fabaceae such as *Thermopsis turcica*, *Senna alata*, and *Bauhinia holophylla* (Cenkci *et al*., 2008; Castro *et al*., 2018; Lara *et al*., 2022).

Results from the current study indicated that all 2,4-D treatments had successfully induced callus from cotyledon explants after eight weeks of culture (Table 1; Figure 3). Next, the control group of cotyledon explants did not form any callus, indicating that auxin 2,4-D is essential for callus induction from cotyledon explants of *C. ternatea*. Meanwhile, a yellowish-brown friable callus was developed at the excision sites of the hypocotyl explants, and 100% callus induction was attainable in all the 2,4-D treatments except for the control group (26.32%) (Table 2; Figure 4). Hence, it is evident that 2,4-D could induce callus from the hypocotyl explants effectively. As in the control group of hypocotyl explants (Figure 5), three phenomena were observed in which some of the explants did not form any callus, some explants formed callus and some formed adventitious roots. The absence of callus was most likely owing to the lack of 2,4-D, a callus-inducing agent. Meanwhile, despite receiving no exogenous auxin, some hypocotyl explants in the control group managed to produce callus (26.32%), demonstrating that endogenous auxins within the hypocotyl explants were adequate to stimulate callus formation without the need of exogenous PGRs. Furthermore, the low frequency of callus formation in the control group was most likely due to the wound response, which included mitosis at the excision sites, resulting in callus formation (Pérez-Francés et al., 1995). This condition is otherwise known as wound-induced

callus formation, in which the wounds would express and upregulate endogenous cytokinin-related genes, which in turn stimulate the callus formation (Iwase et al., 2011a; Iwase et al., 2011b). This finding was in accordance with the study of Sado et al. (2014) on Senna spectabilis (Fabaceae), in which the control group was able to induce callus from hypocotyl explants (25%) despite receiving no exogenous PGRs. Next, the formation of adventitious roots on hypocotyl explants, on the other hand, could be attributable to the de novo organogenesis process that occurred on the damaged tissues. For instance, even in the absence of exogenous PGRs, the damaged area would stimulate and upregulate the genes associated with the root founder cell, resulting in the development of adventitious roots (Liu et al., 2014).

Table 2. The regeneration effects of different concentrations of 2,4-D on hypocotyl explants of *C. ternatea* after 8 weeks of culture in half-strength MS media

2,4-D concentrations (mg/L)	Percentage of callus induction (%)	Callus scoring (Relative value)	Callus texture	Callus colour
0.0 (Control)	25.64 ± 8.57^{b}	0.46 ± 0.16^{b}	Friable, soft, watery, and sticky	Yellowish-brown
0.5	100.00 ± 0.00^{a}	1.80 ± 0.12^{a}	Friable, soft, watery, and sticky	Yellowish-brown
1.0	97.78 ± 2.22^{a}	1.47 ± 0.13^{a}	Friable, soft, watery, and sticky	Yellowish-brown
1.5	100.00 ± 0.00^{a}	1.52 ± 0.13^{a}	Friable, soft, watery, and sticky	Yellowish-brown
2.0	100.00 ± 0.00^{a}	1.31 ± 0.08^{a}	Friable, soft, watery, and sticky	Yellowish-brown
2.5	100.00 ± 0.00^{a}	1.33 ± 0.12^{a}	Friable, soft, watery, and sticky	Yellowish-brown

The data present mean ± SE. Mean values followed by the same alphabet were not significantly different (Tukey HSD test at $p \leq 0.05$).

In the current study, the optimal treatment for callus induction for both the cotyledon and hypocotyl explants was discovered to be 0.5 mg/L 2,4-D. The administration of 0.5 mg/L 2,4-D resulted in 100% callus induction in both the cotyledon and hypocotyl explants. This finding is in parallel with several studies in which the concentration of 0.5 mg/L 2,4-D was discovered to be the optimal treatment for callus induction from cotyledon explants of *Vigna subterranea* L., *Clinacanthus nutans*, *Cicer arietinum* L. (Zaman *et al*., 2010; Konate *et al*., 2013; Phua *et al*., 2016), and hypocotyl explants of *Senna spectabilis* (Fabaceae) (Sado *et al*., 2014). The callus induced from both the cotyledon and hypocotyl explants treated with 0.5 mg/L 2,4-D were morphologically friable, soft, sticky, watery, yellowish-brown in colour, and were appropriate for the establishment of cell suspension culture. As per Frank *et al*. (2000), the morphology of friable callus is the suitable foundation of cell suspension culture because of its soft and easily disaggregated properties into single cells, thereby enabling the continuous and homogenous synthesis of secondary metabolites. Furthermore, friable callus has the qualities of a higher growth rate, making them an ideal choice for establishing cell suspension cultures (Bhatia *et al*., 2015; Bernabé-Antonio *et al*., 2017; Ramulifho *et al*., 2019). Hence, in this study, the induction of friable callus from both cotyledon and hypocotyl explants was found to be suitable for the subsequent establishment of cell suspension cultures from *C. ternatea*.

However, at 0.5 mg/L 2,4-D treatment, the callus scoring was essentially higher for the cotyledon explants (3.03 ± 0.20) than the hypocotyl explants (1.80 ± 0.12), demonstrating that callus development and growth were profoundly related with the type of explants as well as tissue sensitivity to PGRs. This study's observation also revealed that cotyledon explants of *C. ternatea* cultured on half-strength MS medium supplemented with 0.5 mg/L 2,4-D was the optimum explant in inducing callus in comparison to hypocotyl explants. This finding was consistent with Winson *et al*. (2020) in which the cotyledon explants of *Hylocereus costaricensis* (dragon fruit) were found to be the ideal explant for inducing friable callus compared to epicotyl explants. Furthermore, Chan *et al*. (2017) reported that cotyledon explants of *C. ternatea* treated with IBA and NAA resulted in a higher yield of callus as compared to that of hypocotyl explants, which was in parallel with the current study. However, Tiwari and Chaturvedi (2018) demonstrated that hypocotyl explants were the better choice in inducing callus in comparison to leaf explants of *Polygonatum verticillatum* L. as the time required for callus induction was generally shorter and the callus obtained from hypocotyl explants was 1.5-fold higher when compared to the leaf explants under the similar treatment (Tiwari & Chaturvedi, 2018). In short, different species and explant types would respond differently to the same treatment. Hence, explant selection is essential to determine the suitable type of explant capable of greater callus formation.

In the current study, callus induction from both the cotyledon and hypocotyl explants was observed to be lower at concentrations greater than 0.5 mg/L 2,4-D, regardless of being insignificant between treatments. This observation could indicate that concentrations greater than 0.5 mg/L may not be optimal for callus induction for both explants. This finding could also imply that the increase in exogenous auxin concentration was not associated with the increase in callus induction for both the cotyledon and hypocotyl explants of *C. ternatea*. According to Grossmann (2003), low levels of auxin 2,4-D supplementation could increase cell proliferation as well as elongation, thereby resulting in callus development. However, 2,4-D at elevated concentrations can function as a systemic herbicide for weed management and eliminating broad-leaf (dicots) weeds (Grossmann, 2003; de Arcaute *et al*., 2016). This occurrence thereby revealed that 2,4-D does not support callus induction at high doses. Elevated 2,4-D concentrations had been proven to imply a negative effect on callus induction from the root, internodal, and leaf explants of *Achyranthes aspera* L. (Amaranthaceae), leaf explants of *Viola canescens*, hypocotyl explants of *Senna spectabilis* (Fabaceae) and

Acacia sinuata (Leguminosae) (Vengadesan *et al*., 2000; Sado *et al*., 2014; Sen *et al*., 2014; Khajuria *et al*., 2017). Thus, the current study further suggested that the supplementation of 2,4-D at a concentration beyond 0.5 mg/L could be phytotoxic to both the cotyledon and hypocotyl explants of *C. ternatea*, which lead to a decline in callus development.

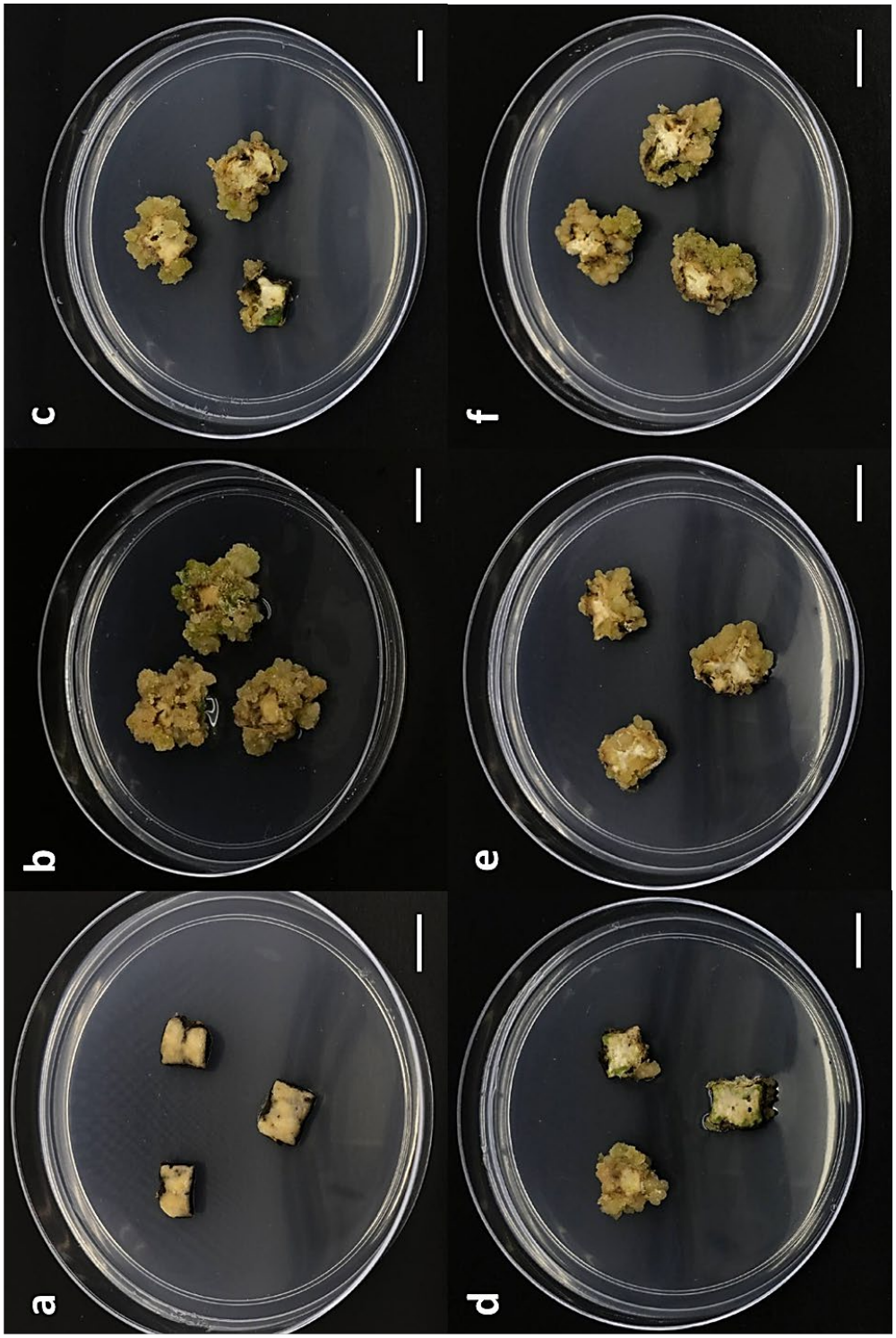

Fig. 3. Callus induction from cotyledon explants at different concentrations of 2,4-D. **(a)** Control, **(b)** 0.5 mg/L, **(c)** 1.0 mg/L, **(d)** 1.5 mg/L, **(e)** 2.0 mg/L, **(f)** 2.5 mg/L. The scale bars represent 1 cm.

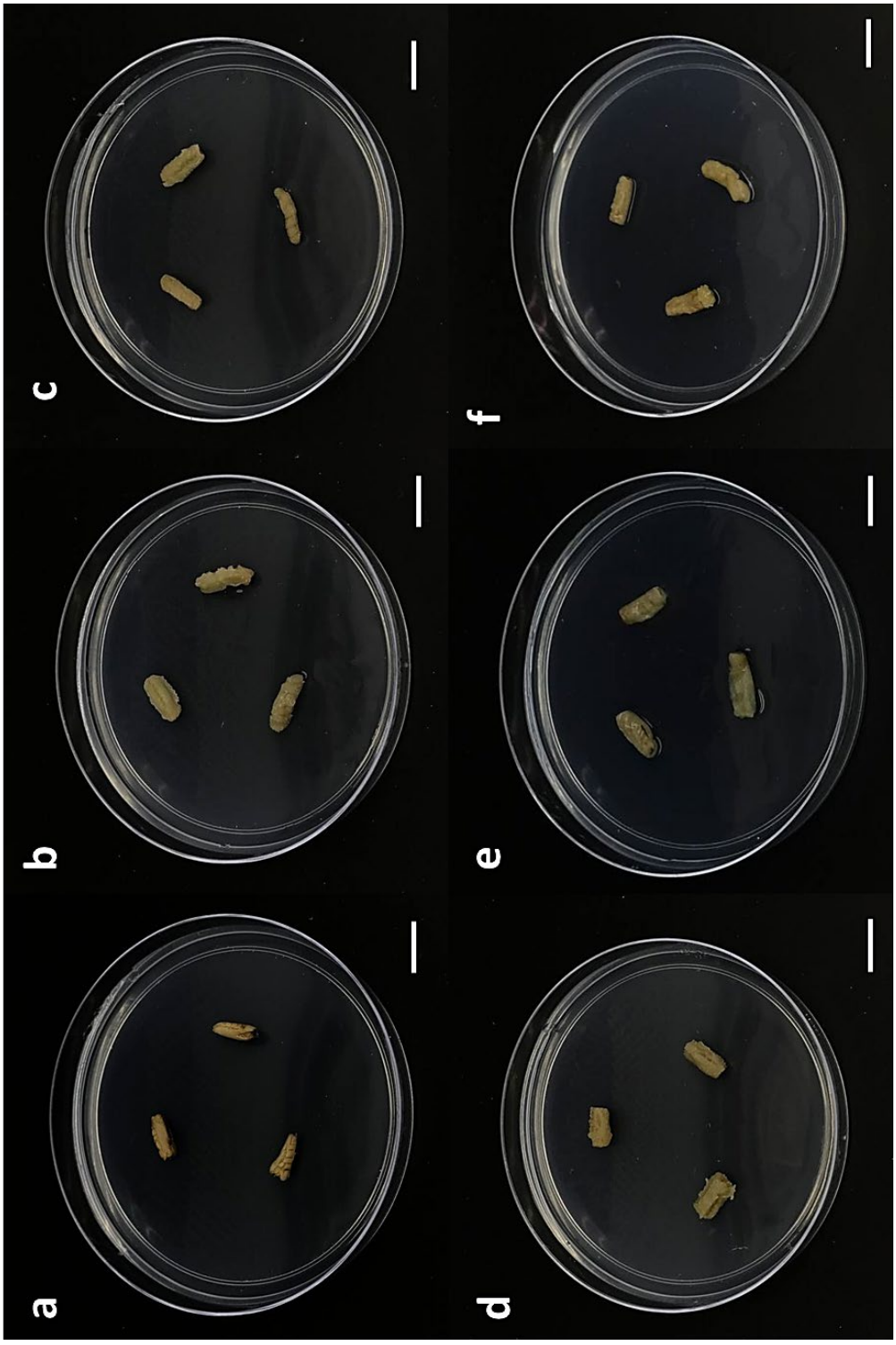

Fig. 4. Callus induction from hypocotyl explants at different concentrations of 2,4-D. **(a)** Control, **(b)** 0.5 mg/L, **(c)** 1.0 mg/L, **(d)** 1.5 mg/L, **(e)** 2.0 mg/L, **(f)** 2.5 mg/L. The scale bars represent 1 cm.

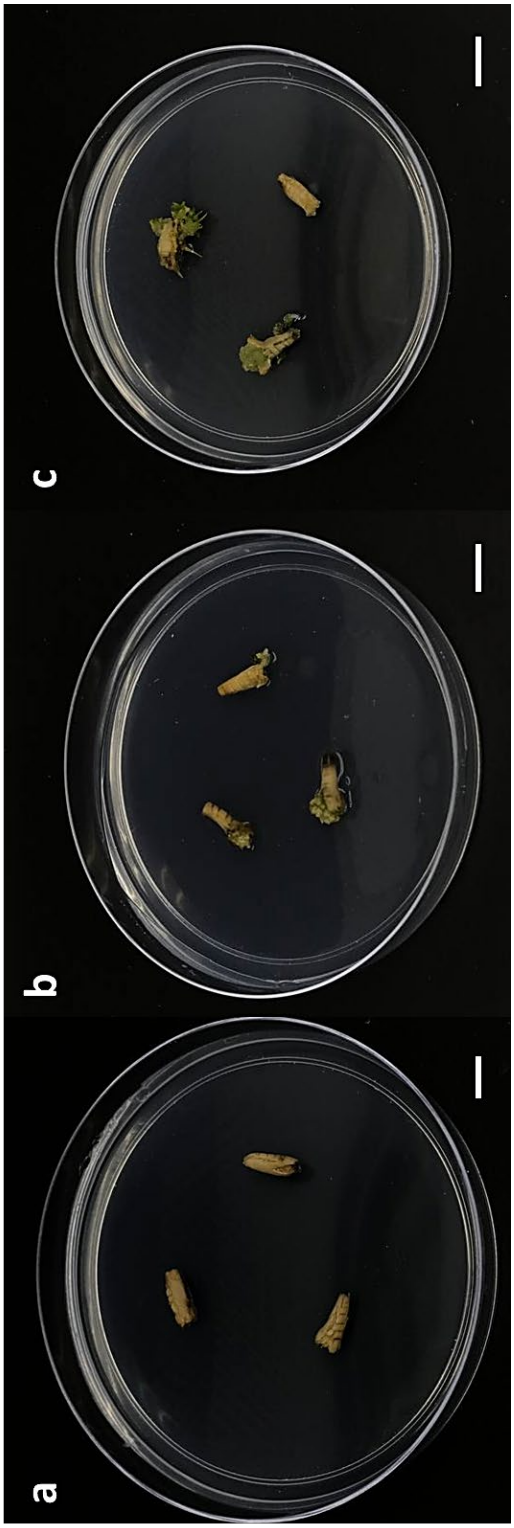

Fig. 5. Hypocotyl explants after 8 weeks of culture under half-strength MS0 semi-solid medium (Control group). **(a)** Explants free from callus formation, **(b)** Explants with callus formation, **(c)** Explants with callus and adventitious roots formation. The scale bar represents 1 cm.

Furthermore, the excision sites of all the cotyledon explants turned brown in colour, whereas the cotyledon explants that were initially green turned yellow or white after 8 weeks of culture (Figure 3). These occurrences were likely due to the underlying oxidative stress upon cutting, which is also known as oxidative browning, a typical problem encountered in plant tissue culture. The issue of oxidative browning arises due to the buildup and oxidation of phenolic chemicals in tissue and culture media, which can ultimately prompt slow growth, cell damage, or even death (Krishna *et al*., 2008; Jones & Saxena, 2013). Subject to that, Jones and Saxena (2013) and Byeon *et al*. (2015) proposed that the addition of melatonin as a supplement could resolve such issues alongside stimulating cell growth.

CONCLUSION

In conclusion, the current study reports on the induction of friable callus from sterile cotyledon and hypocotyl explants of *C. ternatea*. The highest callus induction rate (100%) and scoring were achieved in both cotyledon and hypocotyl explants in the treatment of 0.5 mg/L 2,4-D with friable callus texture. This study also identified cotyledon explants to be the suitable explant type for inducing friable callus. The current study thereby serves as a preliminary assessment in inducing friable callus from *in vitro* seedling explants of *C. ternatea*. Future work can include treatments with other plant growth regulators for optimal production and proliferation of callus cells suitable for the establishment of cell suspension cultures for the production of novel secondary metabolites linked to the mammalian neuroprotective mechanism. Also, browning of explants could also be further studied via the addition of melatonin or activated charcoal.

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